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DNA GENETIC DIVERSITY OF *VETIVERIA ZIZANIOIDES* (POACEAE)

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ABSTRACT

Seventeen world wide accessions of *Vetiveria zizanioides* and six accessions of putative *V. nemoralis* from Thailand were analyzed by the use of random amplified polymorphic DNAs (RAPDs). A vetiver accession from Panamá was found to be the most distinct. All of the putative *V. nemoralis* from Thailand clustered together and the remaining *V. zizanioides* accessions formed a tightly clustered group. Within *V. zizanioides*, several accessions were found that differ from the commonly utilized "Sunshine" cultivar. These new vetiver germplasm sources will be evaluated in replicated test plots and potentially new lines of germplasm can be utilized for soil erosion control in the tropics.

KEY WORDS: geographic variation, vetiver, *Vetiveria nemoralis, V. zizanioides*, Poaceae, random amplified polymorphic DNAs, RAPDs

Vetiver (*Vetiveria zizanioides* [L.] Nash) has been utilized in many parts of the world to control soil erosion. Hedges of the non-seeding vetiver provide an effective living dam against erosion (NRC 1993) and this technique is now in use in more than 100 countries. The origin of the non-seeding vetiver is not known. However, *V. zizanioides* seems to have originated in the area from India to Vietnam and its fragrant roots have been used for centuries for mats and perfumes (NRC 1993).

Kresovich *et al.* (1994) reported on clonal variation of vetiver using random amplified polymorphic DNAs (RAPDs). They found RAPD patterns were very stable within clones and were able to distinguish between various vetiver accessions. Adams & Daffom (1998) examined 121 accessions of vetiver and found that 86% of these appeared to be from a single clone (no variation in the DNA examined). This work was expanded in Adams *et al.* (1998) to include closely related genera (*Chrysopogon* and *Sorghum*). Since these studies, we have found several other genotypes of vetiver and in the present paper we compare these genotypes with six putative *V. nemoralis* Camus accessions from Thailand.

MATERIALS AND METHODS

Specimens were collected as given in Table 1. The leaf samples were shipped desiccated in silica gel (Adams *et al.* 1992). The DNA from vetiver is not preserved well in either fresh or air dried leaves. Interim preservation of the leaves in silica gel is necessary. Upon receipt, all the materials were frozen until analyzed. DNA was extracted using the hot CTAB protocol (Doyle & Doyle 1987) with the addition of 1 % (w/v) PVP and Proteinase (150 µg). Grinding in hot CTAB (60° C) in a hot mortar and pestle was somewhat effective for some accessions, but most accessions yielded larger molecular weight DNA and greater yields when the tissue was ground in liquid nitrogen and then placed in hot CTAB.

PCR was performed in a volume of 15 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 µM primers, 0.3 ng genomic DNA, and 0.6 unit of Taq DNA polymerase (Promega). A control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination.

The following fifteen, ten-mer primers (numbers from U. British Columbia project) were used: 131, GAA ACA GCG T; 134, AAC ACA CGA G; 153, GAG TCA CGA G; 184, CAA ACG GAC C; 212, GCT GCG TGA C; 218, CTC AGC CCA G; 234, TCC ACG GAC G; 239, CTG AAG CGG A; 249, GCA TCT ACC G; 250, CGA CAG TCC C; 265, CAG CTG TTC A; 268, AGG CCG CTT A; 327,

ATA CGG CGT C; 338, CTG TGG CGG T; 347, TTG CTT GGC G. These primers gave several bright bands, did not have any false bands (in the controls) and were proved to be reproducible in replicated analyses. DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). The thermal cycle was: 94° C (1.5 min) for initial strand separation, then 40 cycles of 38° C (2 min), 72° C (2 min), 91° C (1 min). Two additional steps were used: 38° C (2 min) and 72° C (5 min) for final extension. Amplification products were analyzed by electrophoresis on 1.5% agarose gels and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. pGEM DNA (Promega) was used as a molecular weight marker. The RAPD bands were scored by molecular weight and assigned a code based on primer number prefix and molecular weight category. In addition, the RAPD band intensity was scored as: 0 = no band; 4 = faint; 5 = medium; 6 = bright band, in reference to a gray tone standard (Adams & Demeke 1993). It might be noted that analyses using simple 0 = absent and 1 = present scoring gave very similar results, except the eigenroots were not as strongly loaded on the first few axes, implying that the information content was less than when the 0 - 6 scale was used. In replicated analysis, we have found that the relative band intensity is very reproducible in our lab. In our RAPD analyses, every primer generated at least one very bright band (level 6). Over the past several years we have screened over 250 primers and selected about fifteen primers that we use routinely. Any primer that did not generate at least one level 6 band (very bright) was not used in the analyses. In addition, if the PCR amplification does not result in at least one level 6 band, the sample is re-analyzed in triplicate. Invariably, upon re-amplification all three re-analyses resulted in at least one level 6 band. The brightest of the triplicate samples is then re-electrophoresed with the other samples. This iterative approach results in obtaining a set of very similar amplifications for each sample. Thus, the relative intensities are preserved.

Several factors may be responsible for the presence of faint bands: single copy DNA for faint bands vs. multiple DNA copies for bright bands; tertiary folding of DNA with cross bonding making the DNA less amenable to PCR amplification; and competitive interactions between bands for TAQ enzymes and substrates during amplification.

These data were coded into a matrix of taxa by character values. Similarity measures were computed using absolute character state differences (Manhattan metric), divided by the maximum observed value for that character over all taxa (= Gower metric; Gower 1971; Adams 1975). Division by the character state range was tried and found to be less informative than using the maximum observed character value (*i.e.*, including zero in the range). Principal coordinate analysis (PCO) of the similarity matrix follows Gower (1966) by program PCO3D (available for PC computers from RPA).

Table 1. Vetiver accessions used in this study. Those accessions marked with asterisk (*) will be planted in test plots for evaluation when possible.

Code	Accession #	Lab #	Species	Source
*SS	VET-MRL-001	7749	*V. zizanioides*	*cv.* 'Sunshine', Louisiana, USA
*B1	VET-NS-001	8339	*V. zizanioides*	Songkla 1, Thailand
*B2	VET-NS-002	8340	*V. zizanioides*	Surat Thani, Thailand
*B3	VET-NS-003	8341	*V. zizanioides*	Sri Lanka, via Thailand
B4	VET-NS-005	8342	*V. nemoralis*	Kamphaeng Phet, Thailand
B5	VET-NS-006	8343	*V. nemoralis*	Loei, Thailand
B6	VET-NS-007	8344	*V. nemoralis*	Nakon Sawan, Thailand
B7	VET-NS-008	8345	*V. nemoralis*	Prachuap Khiri Khan, Thailand
B8	VET-NS-009	8346	*V. nemoralis*	Ratchaburi, Thailand
B9	VET-NS-010	8347	*V. nemoralis*	Roi Et, Thailand
*ML	VET-TGML-001	8244	*V. zizanioides*	*cv.* 'Malaysia', Malaysia via Spain
*KR	VET-TGKN-003	8246	*V. zizanioides*	*cv.* 'Karnataka', Malaysia via Spain
SH	VET-TGSB-004	8247	*V. zizanioides*	*cv.* 'Sabah', Malaysia via Spain
SB	VET-TGBB-005	8248	*V. zizanioides*	*cv.* 'Sabak Bernam', Malaysia via Spain
PT	VET-TGPB-006	8249	*V. zizanioides*	*cv.* 'Parit Buntar', Malaysia via Spain
*AV	VET-TGAVC-002	8245	*V. zizanioides*	*cv.* 'AVC', Am. Vet. Corp., via Spain
*HF	VET-MB-01	8029	*V. zizanioides*	*cv.* 'Huffman', Florida, USA
*CP	VET-LW-0001	8048	*V. zizanioides*	*cv.* 'Capitol', Louisiana, USA
*SL	VET-RN-001	7951	*V. zizanioides*	Colombo, Sri Lanka
*MA	VET-IMZ-AGA	8349	*V. zizanioides*	Lilongwe, Malawi
*CR	VET-JM-PV1	8076	*V. zizanioides*	Puerto Viejo, Costa Rica
PB	VET-RGG-PA-B	7720	*V. zizanioides*	western site B, Panamá
*ZM	VET-SJC-2	7775	*V. zizanioides*	Zomba, Malawi

RESULTS AND DISCUSSION

The RAPD amplifications resulted in 217 bands. A minimum spanning tree (Figure 1) shows that three major groups are present in the data set: "Sunshine" vetiver, the six putative *Vetiveria nemoralis* from Thailand (B4-B9), and the single Panamá B accession from Panamá. Note that AV (American Vetiver Corp.) and KR (*cv.* Karnataka from Malaysia) show the greatest differences in the "Sunshine" vetiver complex. All of these accessions are non-fertile.

Principal coordinate analysis (PCO) of this similarity matrix removed 69.4% of the variance among the accessions by nine eigenroots. These eigenroots accounted for 20.4, 10.0, 8.2, 6.7, 5.6, 5.2, 4.8, 4.3, and 4.2% of the variance. The eigenroots appear to asymptote after the 5th root. A 3-d ordination reveals that coordinate 1 separated the putative *Vetiveria nemoralis* (Thailand) from all the other accessions (Figure 2). The second axis seems to separate the *V. zizanioides* from Thailand (B1-B3) from other accessions. The Panamá B (PB) accession was separated by the third axis (Figure 2). Clearly, the accessions of *V. nemoralis* from Thailand form a group in both Figures 1 and 2, and this indicates that this group may warrant some taxonomic recognition.

In order to more clearly discern the inter-relationships among the *Vetiveria zizanioides* accessions, the putative *V. nemoralis* accessions and the Panamá B accession were removed from the data set and a new PCO was performed. This PCO resulted in removing 82.1% of the variance among accessions by the first nine eigenroots: 20.0, 12.0, 10.2, 9.3, 7.7, 7.0, 6.0, 5.1, and 4.8%. The first ordination (Figure 3) shows that the "Sunshine" accession occupies a central position with the three Thailand accessions (B1-B3) ordinated away from the main portion of the vetiver accessions. Several Malaysian accessions tended to cluster as a group on the left (Figure 3). The second coordinate shows the unique nature of one of the Malaysia accessions (ML in Figure 3) and the Karnataka (KR) accession. It should be noted that the relationships shown in the minimum spanning network (Figure 1) were unaffected by removing the *V. nemoralis* and Panamá B accessions, because they were not most similar to any of the *V. zizanioides* accessions (data not shown).

Accessions AV (American Vetiver Corp.) and KR (Karnataka, Malaysia) were heavily loaded onto coordinates 4 and 5, respectively. Ordination using axes 1, 4, and 5 reveals that these OTUs are quite distinct (Figure 4). Note particularly that AV is not clustering close to Sunshine (SS), in contrast to Figure 3. This distant relationship to SS is, of course confirmed in the minimum spanning network (Figure

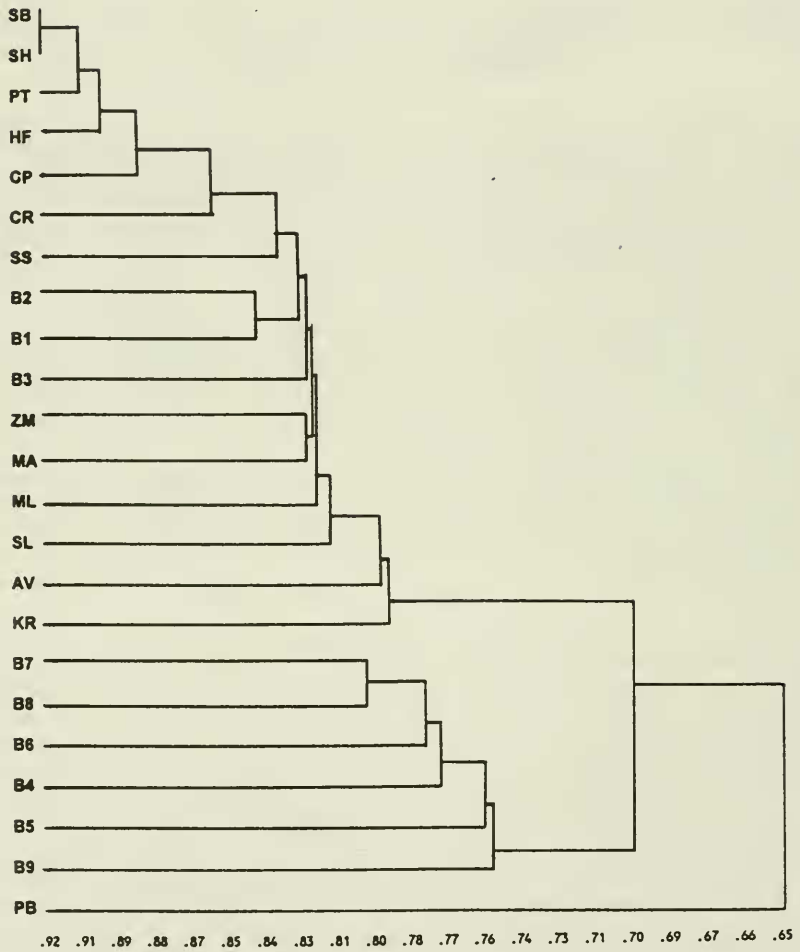

Figure 1. Minimum spanning network for 23 vetiver accessions based on 217 RAPD bands. See Table 1 of code identifications.

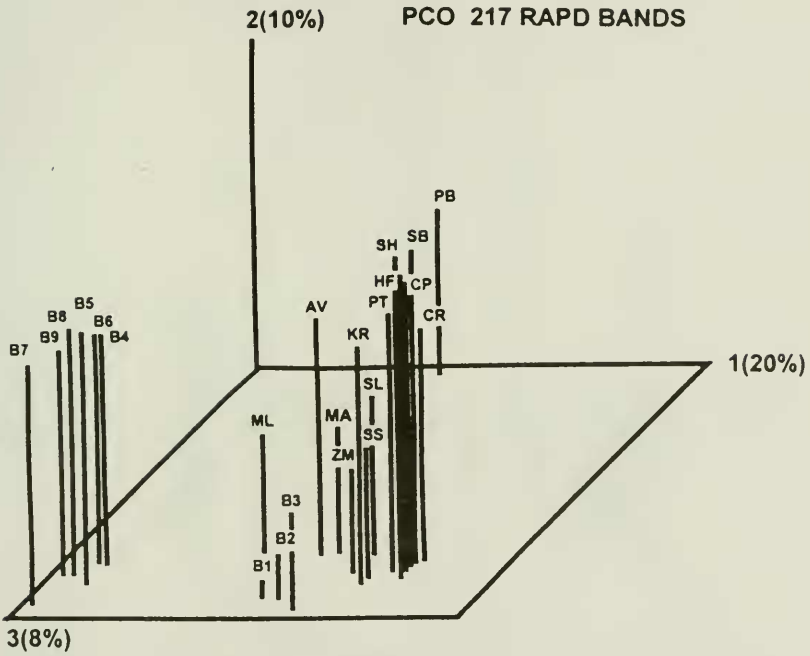

Figure 2. Principal coordinate analysis of the 23 vetiver accessions. Note that accessions B4-B9 are all putative *Vetiveria nemoralis* from Thailand. See text for discussion. See Table 1 of code identifications.

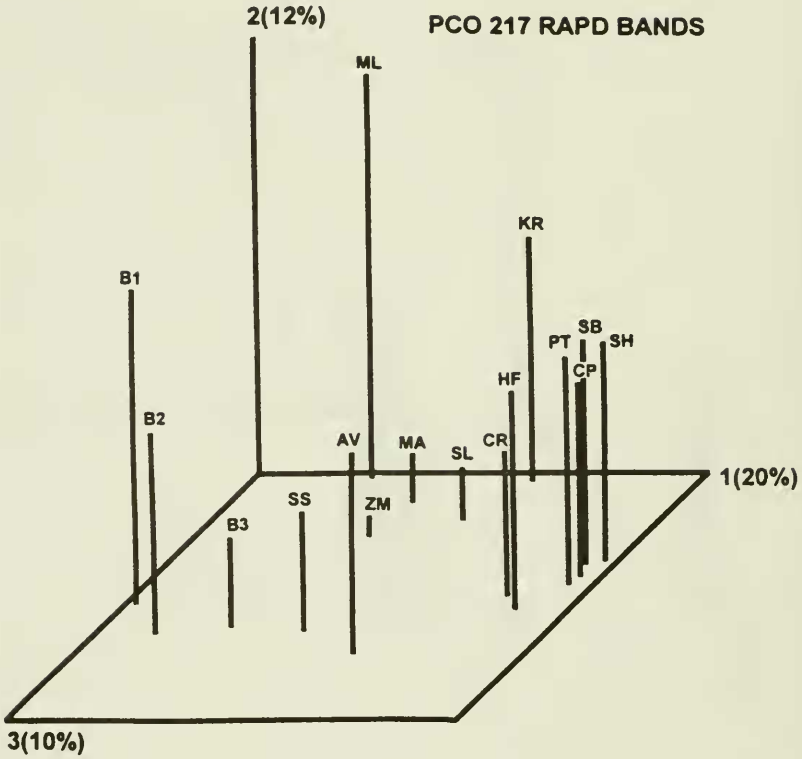

Figure 3. PCO of sixteen, non-seedy vetivers. Note the divergence of the Malaysian accession (ML) and that the Thailand vetivers (B1-3) cluster well with Sunshine (SS). See Table 1 of code identifications

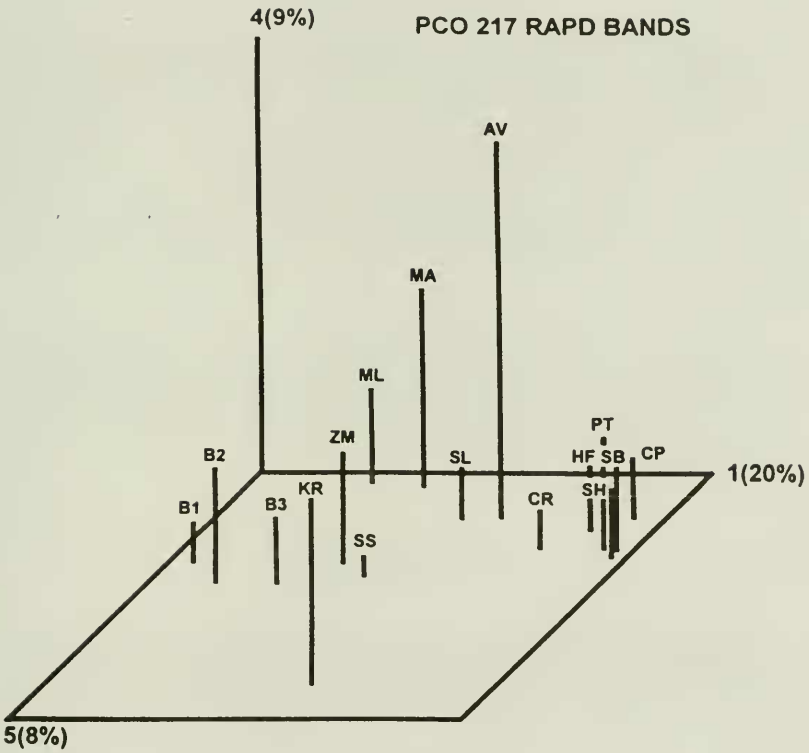

Figure 4. PCO of the sixteen, non-seedy vetivers mapped onto coordinates 1, 4, and 5. Notice the divergence of AV (American Vetiver Corp.) and KR (Karnataka, Malaysia) from the other vetivers. See Table 1 of code identifications

1). It should be noted that there is often distortion when only three axes are used in ordination. In this case, it takes a separate ordination utilizing axes 4 and 5 to portray the variation.

Several points are relevant from this study. New sources of germplasm should be accessioned from the Thailand materials (B1-B3). The Thailand *Vetiveria nemoralis* accessions should be further investigated as to their taxonomic status (species or infraspecific taxon?). In contrast to the previous work (Adams *et al.* 1998), this more robust DNA analysis (217 bands) shows the AV (American Vetiver Corp.) accession to be a source of germplasm that is quite distinct from Sunshine. The Panamá B plants need to be more thoroughly taxonomically investigated. Some of the accessions are so closely related that only one type should be included in test plot evaluation (*e.g.*, SB, SH, PT, HF, see Figures 1, 3), if time and money are constraints.

In order to diversify the current germplasm, we plan to establish test plots in several countries utilizing the following accessions: Sunshine (SS),Songkla (B1), Surat Thani (B2), Sri Lanka (B3) via Thailand, Malaysia (ML), Karnataka (KR), American Vetiver Corp. (AV), Hoffman (HF), Capital (CP), Colombo, Sri Lanka (SL), Costa Rica (CR), and Zomba, Malawi (ZM).

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